

Observation of cavitation and water-refilling processes in plants with X-ray phase contrast microscopy

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Abstract With the spatial coherence of X-rays and high flux and brightness of the 3rd generation synchrotron radiation facility, X-ray phase contrast microscopy (XPCM) at Shanghai Synchrotron Radiation Facility (SSRF) can provide high resolution dynamic imaging of low electron density materials in principle. In this paper, we investigated the cavitation and water-refilling processes in rice and bamboo leaves utilizing XPCM at SSRF. The occurrence of xylem cavitation was recorded *in vivo*. The study also revealed that under different dehydration conditions, cavitation occurs in different degrees, and therefore, the refilling process is different. The results demonstrate that SSRF can provide high enough fluxes to study dynamic processes in plants in real-time, and XPCM is expected to be a promising method to reveal the mechanisms of cavitation and its repair in plants nondestructively.

Key words X-ray phase contrast microscopy, Synchrotron radiation, Cavitation/embolism, Water transportation

1 Introduction

Long-distance water transport plays a crucial rule in the survival of plants, and it is also an important issue in plant physiology and eco-physiology. As early as the 18th century, the argument as to the mechanism of water ascent in higher plants had emerged and it has puzzled plant physiologists and physicists for several centuries. In the past few decades, Cohesion-Tension (C-T) theory is widely accepted to explain the mechanism of water transmission in trees. However, Zimmermann pointed out that due to the existence of cavitation and embolism, the water columns in xylem vessels are not continuous^[1], which challenges the basis of C-T theory. Then, xylem cavitation/embolism has become a hot topic in plant physiology and ecology in the world. However, the mechanism of its occurrence remains unclear, due to limitations in the microscopic techniques. Since last century, researchers have carried out many theoretical analysis or

models^[2-4] or even experimental studies^[5-9] in this field. Lewis *et al.* observed the development of emboli in the tracheids of *Thuja occidentalis* L. by optical microscopy *in vitro*^[7].

Nowadays, many controversial viewpoints about the methods and results arose in this field, because most of the experimental methods require destructive sampling, thus the results are not convincing. For example, in the optical microscopy method, you need to cut a section of the stem or other part of trees or plants as a sample, then slice it into ultra-thin pieces and finally observe the slices under microscopes while dehydrating or refilling. Thus the results become distorted and unconvincing which might not reflect the original status in plants. Nowadays, there are also nondestructive or minimally invasive methods, for example the Acoustic Emission method (AEs) and Nuclear Magnetic Resonance (NMR) imaging. However, the NMR method has low spatial and temporal resolution, which is not high enough to observe a specific xylem vessel.

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Furthermore, it has high requirements for the sample, such as good toughness and large vessels, and it also requires a specimen small enough to fit the sample chamber. The AE method is only used to detect the occurrence of a cavitation event, in which the acoustic emission signals are usually weak, and they are always disturbed by the noise of the instrument itself or the surrounding environment, so it is hard to detect the true signals. It is urgent for a new suitable method to nondestructively observe the inner structures of vessels with higher resolution in real time.

Compared with the destructive and restrictive methods mentioned above, X-rays with their unique penetrating character have great advantages for nondestructive detection. Since Röntgen discovered X-rays in 1895, the applications of x-ray have attracted much attention from researchers all over the world^[10]. At the end of the last century, X-ray propagation-based phase-contrast imaging was proposed for investigation of weakly absorbing materials^[11]. From then on, this method has been applied in a varied range of research fields, such as biology, medicine, materials and many other important fields. In order to meet application requirements, great improvements have been made in applying the propagation-based X-ray phase-contrast imaging method^[12–15] based on both laboratory X-ray source systems and synchrotron radiation facilities. The advantages of a synchrotron radiation facility include higher flux and brightness, as well as superiority in spatial and temporal resolution, without slicing or injecting reagents or other special sample preparations. Considering that the xylem cavitation/embolism and refilling processes happen in a short period of time, X-ray phase contrast microscopy (XPCM) based on synchrotron radiation is an ideal technique to investigate the dynamic processes nondestructively in plants, which are mainly composed of low-Z elements. Lee *et al.* have tried to employ X-ray micro-imaging to the water-refilling process in bamboo leaves intending to understand the mystery of water transport^[16,17]. In this paper, a wiggler source beamline—X-ray Imaging and Biomedical Application Beamline (BL13W1) in Shanghai synchrotron radiation facility—was used to investigate the cavitation and water-refilling processes in plants (both rice and bamboo leaves) by X-ray

phase contrast microscopy.

2 Methods and materials

2.1 Experimental setup

BL13W1 is one of the initial beamlines of SSRF, in which in-line setup is employed to realize X-ray microscopy in phase contrast. X-ray in-line phase contrast microscopy has a simple layout and moderate requirement on the beam chromatic coherence compared to X-ray interferometry and holography.

The factors having effect on imaging quality of the in-line phase contrast microscopy have been systematically investigated^[18,19], and the experimental parameters were optimized accordingly. Shown in Fig.1 is the setup of BL13W1, in which a wiggler source is used to radiate X-rays with large energy range from 8 keV to 72.5 keV. The flux density of BL13W1 is 2.2×10^{10} phs/s/mm²@20 keV@200 mA, which is high enough for dynamic imaging at the sample stage 34 m downstream the source. The low emittance of 3.9 nm-rad at SSRF ensures the high brilliance for imaging in phase contrast. X-ray CCDs with a pixel size from 0.37 to 24 microns and field of view from 0.74 to 50 mm are equipped to record the X-ray images statically or dynamically.

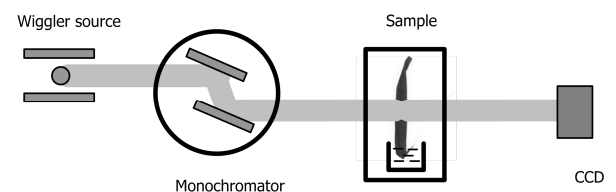


Fig.1 Schematic diagram of BL13W1 at SSRF.

2.2 Experimental parameters

To meet the requirements on high resolution and suitable exposure time, an X-ray CCD from HAMAMATSU was used in the experiments, which has two optional modes with resolutions of 4.5 μm and 1 μm . For the higher spatial resolution camera lens, an exposure time of 5–10 s is needed, which is too long to observe the dynamic phenomenon in living plants. For the 4.5 μm camera lens, the exposure time reduced obviously, only 1–2 s or even hundreds of milliseconds, which is very appropriate for the dynamic experiment in this study. At the same time,

the structures to be observed in these experiments are ten microns to several tens of microns, and 4.5- μm resolution is suitable. Therefore, a CCD with a resolution of 4.5 μm was selected. And the resolution is high enough to visualize the interested structures in the experiments and the exposure time is also acceptable. In the cavitation experiments, a 150 Watt High Pressure Sodium Lamp (HPSL) was used to simulate sunlight, with initial lumens of 13500 lm and mean lumens of 12150 lm.

During the experiments, photon energy of 15 keV, sample-detector distance of 9 cm and exposure time of 600 ms, were selected.

2.3 Sample preparation

Two kinds of plants, bamboo (*CV.Ventricousinternode* was used in this study) and rice leaves, were employed as samples to investigate the cavitation and related repair processes dynamically. The targets of imaging were dynamic behaviors in xylem vessels in plants, the vessel diameter is ranging from several microns to tens of microns. Rice is a kind of monocotyledon and annual herb plants while bamboo is a kind of perennial plants. Both rice and bamboo belong to Gramineae and have lignified leaves, expecting to have high contrast under X-rays.

Before the observation of xylem cavitation experiments, the whole rice plant was immersed in water for several hours to make sure that the leaves were fully hydrated, then one piece of the leaf was cut off from the plant at the joint of the leaf blade and sheath, and finally the leaf was fixed on the sample stage for the XPCM experiments. Meanwhile, the HPSL was fixed 30 cm away from the sample, with an angle of about 45° between the rice leaf and the HPSL, acting as a simulated source of sunlight to accelerate cavitation occurrence of transpiration and generation.

Before the observation of the water refilling experiment, the leaves were firstly cut off at the joint of the leaf blade and sheath, and then dehydrate in the air at least several hours, meanwhile air enters leaves from the cut and cavities generate in the vessels.

3 Results and analysis

3.1 Cavitation processes

3.1.1 The changes of inner-vessels

An image sequence was taken to reveal the xylem cavitation process. Fig.2 shows the cavitation processes that occurred at about 11 min after the irradiation of the HPSL. Shown in Fig.2(A) is the structure before cavitation while Fig.2(B) is the structure after cavitation occurred. The exposure time of Figs.2(A) and 2(B) are both 600 ms. The two images are recorded in temporal sequence, and the acquisition time interval is 1160 ms. As indicated by the arrows, the vessels in Fig.2(B) are clear, while that in Fig.2(A) are indistinct. This is because that vessels in Fig.2(A) are full of water which blurred the XPCM imaging contrast of vessel walls, while that in Fig.2(B) are full of cavities which enhanced the contrast instead. This implies that cavitation occurred in the vessel. From Fig.2(B), the wall between two adjacent vessel elements could be clearly identified as indicated by the arrow of the lower one. The instantaneous occurrence of xylem cavitation was recorded *in vivo* for the first time at home, which will be very useful for the study of cavitation and embolism in intact plants. XPCM is expected to be a helpful method to understand the mechanism of the cavitation happening in plants under different stresses.

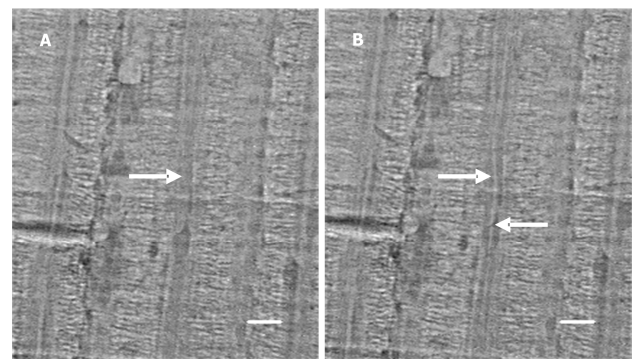


Fig.2 XPCM images of the cavitation process under drought stress, in which A, B are two images recorded in sequence with an exposure time of 600 ms, scale bar=50 μm .

3.1.2 The changes of other parts of the leaves

Due to the limited visual field of the detector, we cannot obtain the dynamic process of the cavitation of the whole sample at the same time. Therefore, in order to get the overall cavitation changes of the whole

blade, images of several other positions of the sample were also collected before and after the real-time acquisition. And during the experiment, high pressure sodium lamp was also used as simulating sunlight.

Results showed that, after tens of minutes or even a few hours of simulated sunlight illumination, most parts of the leaves undergone cavitation. Fig.3 shows the tissue changes of rice leaf in different parts before and after the 1 h cavitation experiment. In region 1, a distinct cavitation occurred in the vessel which is pointed by the arrow. In region 2, more cavitations occurred, and we can even distinguish the two adjacent vessel elements (they are pointed by the arrows). The Wedge-shaped structure connecting two vessel elements is also very clear. Meanwhile, the sample has undergone obvious contraction after the cavitation experiment, and we can obtain more microstructure information of the leaf with the same CCD visual field. It also can be verified by the images of region 3 which show that after the cavitation experiment, the contrast of the vessels is enhanced, and the contraction effect is more obvious.

3.2 Water refilling processes

3.2.1 Rice leaf

During the sample preparation for the rice leaf water refilling experiment, the rice leaves were firstly cut off at the joint of the leaf blade and sheath, then dehydrated in the air for 3 h. During the time, we suppose that air has entered the leaves from the cut and cavities generated in the vessels of the leaves finally. And we will verify this supposition in the following. The leaf was fixed vertically on the sample stage, with the cut end of the sample immersed in water to rehydrate. Meanwhile, the leaf was observed in real time using X-ray phase contrast imaging. At the same time, the inner structures of embolized xylem vessels and the water refilling processes were recorded in temporal sequences.

During rehydrating, 60 images of the dynamic process of water refilling in a rice leaf were obtained, in which water transport in the vessels of the rice leaf was observed, as shown in Fig.4. Fig.4 shows two frames of the dynamic process of refilling, while the first image of the refilling sequence (Fig.4A) shows

the structure before refilling and the last image of the refilling sequence (Fig.4B) shows the structure after refilling. From Fig.4, it is obvious that some of the vessels in the last image have worse phase contrast than that in the first image (Fig.4A).

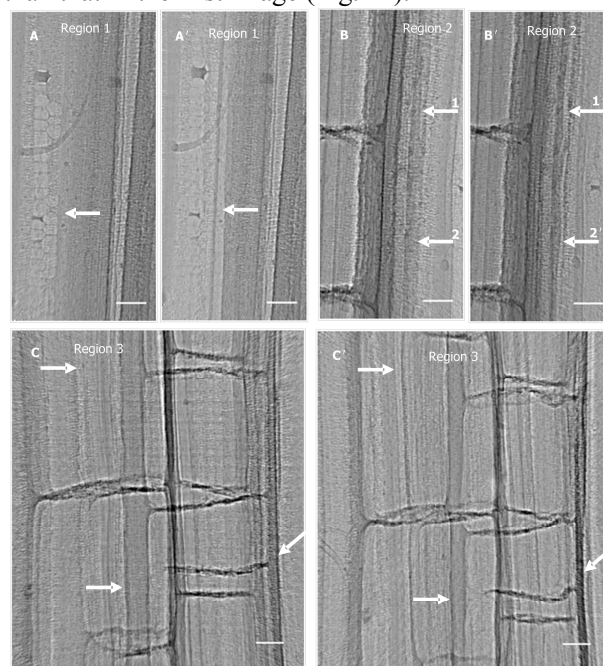


Fig.3 Microstructural changes in different parts of the vessels and its surrounding tissues before and after nearly 1 h water stress. Scale bar=100 μ m.

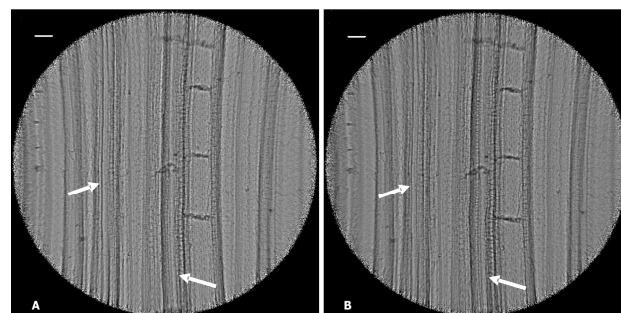


Fig.4 Frames of water refilling process of rice leaf. (A) the first frame, (B) the last frame (scale bar=100 μ m).

This would suggest that vessels in the last image are essentially full of water, while those in the first image are full of cavities. Fig.5 gives the comparison of a certain area of magnified vessels before and after refilling, from which a difference in contrast can be easily distinguished. In Fig.5A, the vessel pointed to by the arrow is embolized before refilling occurs, the air in the vessel could enhance its relative contrast to the surrounding tissues. Shown in Fig.5B are the vessels refilled with water, which blurred the vessel contrast relative to the surrounding tissues. Besides the

differences in phase contrast, the leaf became swollen in the vertical direction of the veins after refilling, because the diameter of xylem vessels in plants varies with the moisture content of the surrounding matrix^[20].

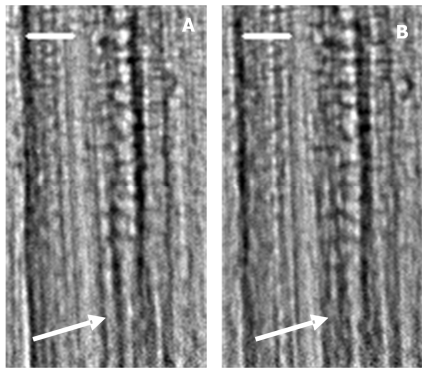


Fig.5 Magnified images for comparison (scale bar=50 μm), where (A) before refilling, (B) after refilling.

3.2.2 Bamboo leaf

Water-refilling experiments on bamboo leaves were also carried out. The preparation and operation was the same as that for rice leaves, the only difference was the dehydration time which was about 1 day. It was much longer than the rice leaf experiment. The whole refilling process was recorded in sequence, in which a total of 196 frames were recorded with an exposure time of 2 seconds for one frame. The frame No.60 and No.100 are shown in Fig.6, in which the arrows show the “wave front” of refilling water. We could see that the vessels were still visible in good contrast relative to the surrounding tissues after refilling, because they were embolized permanently. Therefore, during refilling, most vessels couldn’t transport water any more. The water could rise only along the dehydrated mesophyll, which is located between the upper and lower epidermis. So the refilling water in the leaf was rising as a whole rather than through a specific vessel for transportation.

Embolization is the leading cause of death of plants. If permanent embolism occurs in a certain percentage of vessels, the plant will die. There are already some studies trying to make out the relationship between embolism and permanent wilting^[21]. However, previously there has been no suitable method which could give the accurate relationship. Up to now, the only effective way to determine whether a plant which has undergone drought stress will survive or die is to observe whether

the wilted plant will recover after refilling for a period of time. The results reported in this letter may open a new way for the investigation of drought-resistant trees and crops. It might valuably be used to investigate different lethal thresholds of various plants under different drought stresses.

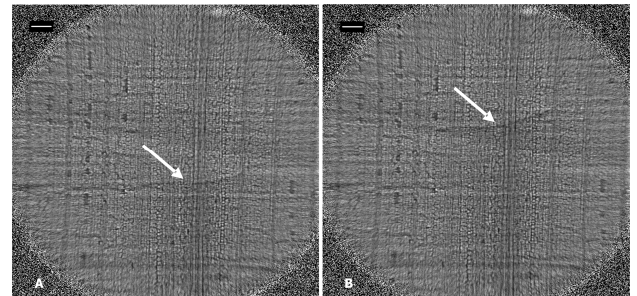


Fig.6 Frames of water refilling process in bamboo leaf. (A) frame of No.60, (B) frame of No.100 (scale bar=100 μm).

4 Conclusion

In conclusion, the experimental results presented here and carried out at SSRF demonstrate that cavitation and water-refilling processes in plants can be visualized *in vivo* with X-ray phase contrast microscopy. As we know, embolism repair is affected by many factors, but the most important element is the hydraulic conductivity in plant vessels which determines the extent of embolism and its repairing mechanism. Among all the available techniques, XPCM appears to be the most promising one to investigate this phenomenon *in vivo*, due to its high spatial resolution and non-destructivity. With the high X-ray flux density available at 3rd generation synchrotrons, it is also possible to obtain the microscopic images with high frame rate. What is more, the low emittance at SSRF ensures the high coherent flux for the imaging of live plant cells via phase contrast.

To sum up, X-ray phase contrast microscopy method opens a potential way for the observation of inner microscopic structures of plants *in vivo*. It is of great significance not only for the investigation of xylem cavitation, embolism and repairing processes in plants, but also for the study of drought-resistant mechanism and lethal threshold of crops and trees.

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